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(54) Title: MELUSIN, A MUSCLE SPECIFIC PROTEIN, AS A DRUG TARGET FOR PREVENTION AND TREATMENT OF HEART FAILURE

(57) Abstract: The present invention concerns non-human transgenic animals as model study for human pathologies, being transgenic for having altered melusin expression. The non-human transgenic animals are to be used as models to study heart pathologies and provide therapies thereof, wherein the heart pathologies are heart failure, and in particular diluted cardiomyopathy.

MELUSIN, A MUSCLE SPECIFIC PROTEIN, AS A DRUG TARGET FOR PREVENTION AND TREATMENT OF HEART FAILURE

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5 ## FIELD OF INVENTION

The present invention concerns non-human transgenic animals as model study for human pathologies, being transgenic for having altered melusin expression. The non-human transgenic animals

10 are to be used as models to study heart pathologies and provide therapies thereof, wherein the heart pathologies are heart failure, and in particular dilated cardiomyopathy.

In a further embodiment the present invention

15 concerns methods for the development of therapeutical approaches for prevention and treatment of hearth failure, in particular dilated cardiomiopathy, by means of using melusin protein and/or nucleic acids encoding for melusin protein, fragments and/or derivatives

20 thereof.

BACKGROUND OF INVENTION

In subjects affected by arterial hypertension, the left ventricle of the heart is subjected to increased mechanical activity in order to pump blood against

25 increased blood pressure. Under these conditions the heart undergoes a compensatory hypertrophy in which cardiomyocytes increase in size as consequence of increased synthesis and assembly of contractile proteins of actomyosin fibrils.

30 Although hypertrophy is compensatory and beneficial allowing the generation of more contractile force, under condition of chronic high blood pressure, additional events might occur that either reduce the efficacy of the hypertrophy response or activate

additional pathways causing cardiac dilation and progressively leading to heart dysfunction and failure.

The identification of the molecular mechanisms involved in the initial cardiac hypertrophy and in the onset of a subsequent defective cardiomyocytes response is a major challenge of the cardiovascular biology and medicine in these days. In fact, understanding such molecular mechanisms can be of great importance to develop therapeutical strategies aimed to fight congestive heart failure, a pathology that, only in the United States of America, affects more that 400.000 peoples every year.

Considerable efforts have been made in the past decade to identify the molecular mechanisms at the cellular level involved in the hypertrophic response of cardiomyocytes. These studies led to a mechanistic model illustrated in Figure 10 in which mechanical stretching induced by hemodynamic overload (1) trigger intracellular mechanosensors (2) that activate intracellular signaling pathways (3) leading to hypertrophy by dual modes: by direct activation of muscle specific genes (4a) and by inducing secretion of neurohumoral and autocrine factors (4b) that in turn act on the cardiomyocytes via specific receptors and signaling contributing to the hypertrophic response.

Among the signaling molecules (point 3 of figure 10) thought to be involved in the cardiac hypertrophy in response to mechanical overload are: the alfa Gq subunit of the heterotrimeric G protein coupled to the beta adrenergic receptors (Akhter et al. 1998), the phospholipase C beta and protein kinase C, acting downstream of the G proteins (Wakasaki et al. 1997), the Calcineurin/NF-AT3 pathway, the Ras cascade including Raf-1 and ERK1/2 MAP kinases, the stress kinases Jnk and p38, the phosphoinositide 3-kinase, the

Jak-STAT pathway (for review see Aoki and Izumo 2001;
Ruwhof and van der Laarse 2000; Hunter and Chien 1999).
These molecules, although very important in inducing
the hypertrophic response, are all acting quite
5   downstream along the signaling pathways.

It is thus clear that identification of the
mechanosensors themselves (point 2 of Figure 10) would
be of great importance, since interference with such
upstream regulatory elements would allow a much more
10 specific control of the hypertrophic response.

The mechanical tension in the muscle is exerted by
the contractile proteins of the cytoskeleton, the
actomyosin fibrils which are physically linked to the
plasma membrane and to the extracellular matrix, via
15 membrane receptors belonging to the integrin family.

In muscles, integrins are preferentially localized
in specific sites known as myotendinous junction and
costamers. These are specific sites were actomyosin
fibrils are connected to the plasma membrane
20 contributing to a correct and stable association of the
contractile machinery to the membrane of the muscle
cells.

Besides transmitting the contractile force across
the plasma membrane, these junctions are also important
25 mechanosensors capable of transmitting signals inside
the cell in response to mechanical stretching. Several
proteins are in fact localized at these sites at the
cytoplasmic face of the plasma membrane and interacting
with integrins. These proteins include paxillin,
30 vinculin, talin, and the tyrosine kinase p125Fak. This
molecular machinery is activated by mechanical
stretching of the cells (for review see Davis et al.
2001; Carson and Wei 2000) and is the best candidate as
the mechanosensing apparatus.

A beta1 integrin isoform (beta1D) that is specifically expressed in striated cardiac and skeletal muscle has been disclosed (Belkin et al 1996). In association with the alpha7 subunit, beta1D forms an

5 heterodimer a7b1D with receptor activity toward merosin (laminin 2) of the extracellular matrix. Functional analysis indicated that beta1D integrin binds both cytoskeletal elements and extracellular matrix ligands with much higher affinity compared to the beta1A

10 isoform present in all non-muscle tissues (Belkin et al 1997) suggesting that beta1D provides a stable actin-laminin interaction across the plasma membrane necessary to support the mechanical tension during muscle contraction.

15 To further define the molecular basis of these functional properties the inventors searched for proteins capable to bind to the cytoplasmic domain of beta1D. Using the two-hybrid screening the inventors isolated melusin, a novel protein selectively expressed

20 in skeletal muscle and heart (Brancaccio et al. 1999; GenBank AF140690; GenBank AF140691).

Sequence analysis of melusin indicated the presence in the amino terminal half of the protein of a tandem repeated cysteine and histidine rich sequence

25 and of putative binding sites for SH2 and SH3 domains. The C terminal half comprises the binding site for the integrin cytoplasmic domain and is characterized by a stretch of acidic amino acid residues binding to Ca^{2+} (Figure 1). Melusin is localized at costamers in

30 correspondence of Z line where also integrins and vinculin are concentrated (Brancaccio et al. 1999).

Melusin, thus likely represents a new intracellular transducer of beta1 integrin function in muscle cells.

DESCRIPTION OF THE INVENTION

The invention concerns non-human transgenic animals as model study for human pathologies, being transgenic for having altered melusin expression. Preferably the human pathology is included in the following group: heart failure, dilated cardiomyopathy, hypertensive cardiomyopathy, hypertrophic cardiomyopathy, congestive heart failure, heart infarct. More preferably the human pathology is dilated cardiomyopathy.

In a preferred embodiment the non-human transgenic animal subjected to experimental hypertension conditions, such as for example surgical constriction of the aorta, pharmacological treatment with hypertensive drugs or high sodium diet, exhibits dilated cardiac hypertrophy and contractile dysfunction and is useful as a study model to provide therapies thereof.

It is a further object of the invention cells derivable from the non-human transgenic animal of the invention. The invention concerns different uses of the cells for the selection of molecules pharmacologically effective in triggering melusin activation.

Another aspect of the invention relates to a method for the preparation of a non-human transgenic animal comprising essentially the steps of i) preparing a non-human transgenic animal carrying an inactivated melusin allele; ii) breeding the parent transgenic animal with another non-transgenic animal; iii) selecting transgenic animals heterozygote for the melusin mutation; iv) breeding of the heterozygote animals to select homozygote animals for the melusin gene mutation.

Another aspect of the invention relates to the preparation of a non-human transgenic animal in which

the melusin gene is inactivated by genetic approaches distinct from homologous recombination, such as for example antisense RNA or RNA interference approaches in which short RNA sequences complementary to melusin transcript or DNA are used to interfere with either transcription or translation of the melusin gene.

In a further embodiment the present invention concerns methods for the development of therapeutical approaches for prevention and treatment of hearth failure, in particular dilated cardiomiopathy, by means of using melusin protein and/or nucleic acids encoding for melusin protein, fragments and/or derivatives thereof.

In a related aspect, the invention provides methods for identifying chemical compounds that are agonists of melusin, being such agonists in the form of peptides or structural analog organic compounds, which consequently can be used for the manufacture of pharmaceutical compositions for the therapy of heart pathologies.

The present invention relates also to the use of melusin for the manufacture of a medicament for treatment and prevention of hearth pathologies in humans; in particular relates to the use of i) melusin protein, peptides, fragments and/or derivatives thereof, ii) nucleic acids encoding for melusin protein, peptides, fragments and/or derivatives thereof for the preparation of pharmaceutical compositions for the prevention and treatment of heart pathologies.

A further aspect of the present invention relates to proteins able to interact with melusin and acting as downstream transducers of the mechano-chemical signal and leading to the activation of genes of the hypertrophy cardiomyocyte program. Chemical compounds acting as agonist toward such proteins can be utilized

as potential drug in the prevention and treatment of hear failure.

According to the present invention, these purposes are achieved by means of the claims which follow.

5   DETAILED DESCRIPTION OF THE INVENTION.

The present invention will now be described in detail with reference to the attached drawings, which are provided purely by way of non-limiting examples and in which:

10   - Figure. 1. Amino acid sequence of Mouse (Mo) and Human (Hu) melusins as deduced from the corresponding cDNAs (murine GenBank AF140691; human GenBank AF140690). Underlined are the cysteine and histidine rich domains (continuous) and the carbossiterminal

15   acidic domain (dotted). Putative binding sites for SH2 and SH3 domains are indicated in bold. The integrin-binding region is boxed. Vertical bars indicate identical amino acids between mouse and human molecules, while double dots are conserved residues.

20   (Brancaccio et al. 1999).

- Figure 2. Genomic DNA construct utilized for the homologous recombination event in ES cells.

- Figure 3. Southern Blot analysis of the ES cells carrying the mutated melusin gene.

25   - Figure 4: Western blot analysis of melusin and integrins expression in wild type and mutant mice.

- Figure 5. Echocardiographic and hemodynamic parameters in wild type (WT) and melusin-null (KO) hearts in basal conditions. Figure 5A, left ventricular

30   end diastolic chamber diameter (LVEDD), left ventricular end systolic chamber diameter (LVESD), interventricular septum thickness in end diastole (IVSD) and left ventricular posterior wall thickness in end diastole (LVPWD). Figure 5B left ventricular

35   weight expressed as mg per gr of body weight (LVW/BW).

Figure 5C, fractional shortening (FS) of left ventricle calculated as [(LVEDD-LVESD)/LVEDD]x100. Figure 5D, left ventricular pressure measured in anesthetized mice by a French high fidelity catheter-tip micro manometer (Millar Instrument).

-       Figure 6. Scheme of the surgical constriction of the transverse aorta (TAC) utilized to induce pressure overload.

-       Figure 7. Left ventricle growth response to transverse aortic constriction in wild type (+/+) and mutant mice (-/-).

-       Figure 8. Echocardiographic parameters in wild type (WT) and melusin-null (KO) hearts of control mice (Sham) or mice subjected to transverse aortic constriction (TAC). Figures 8A, left ventricular end diastolic chamber diameter (LVEDD). Figure 8B, left ventricular end systolic chamber diameter (LVESD). Figure 8C, interventricular septum thickness in end diastole (IVSD) and left ventricular posterior wall thickness in end diastole (LVPWD).

-       Figure 9. Left ventricle growth response to sub-pressor doses of phenylephrine and angiotensin II. Cardiac hypertrophy was evaluated by left ventricular/body weight ratio as indicated on the Y-axis. -/- and +/+ indicate the mutant (black squares) and wild type (empty squares) mice respectively.

-       Figure 10. Diagrammatic representation of the molecular mechanism at the cellular level involved in hypertrophic response of cardiomyocytes.

-       Figure 11. Left ventricle remodeling and function after 2 and 4 weeks from TAC in wild-type (WT, empty bars) and mutant mice (KO, filled bars). Figure 11a: Representative M-mode left ventricular echocardiografic recording of wild-type (upper pictures) and melusin-null (lower pictures) mice. Figure 11b: Left

ventricular end diastolic diameter (LVEDD). Figure 11c: interventricular septum thickness in end-diastole (IVSTD). Figure 11d: percent fractional shortening (%FS) as parameter of left ventricle contractile
5   function. Figure 11e: Representative gross morphology of whole hearts (upper rows) and transversal sections at base level of the left ventricles of wild-type and melusin-null mice after 4 weeks from TAC. §: $P<0.01$ vs Basal; *: $P<0.01$ vs wild-type; °: $P<0.05$ vs wild-type.

10 -   Figure 12. Impaired GSK3β phosphorylation in melusin-null mice in response to TAC by comparison of results between Wild-type (WT, empty bars) and melusin-null mice (KO, filled bars). Left ventricle protein extracts were analyzed by western blotting with
15 antibodies to phosphorylated signaling molecules. Sample loading was controlled using antibodies specific for each protein. The intensity of the bands from two independent experiments with a total of 8 mice for group was measured and relative intensity was
20 calculated after subtraction of basal level in sham operated animals. ERK (Figure 12a), p38 (Figure 12b), GSK3β (Figure 12c), AKT (Figure 12d) are rapidly phosphorylated in response to TAC in wild-type mice, but phosphorylation of GSK3β and AKT were strongly
25 impaired in melusin-null mice. GSK3β was phosphorylated to a comparable level in both wild-type and melusin-null mice 10 and 20 min after IP injection of 2 IU of insulin (Figure 12e). Reduced serine9-GSK3β phosphorylation (Figure 12f) and increased kinase
30 activity (Figure 12g) were detected in melusin-null hearts subjected to 7 days of TAC. °: $P<0.05$ vs wild-type.

The present invention will now be described in relation to some preferred embodiments by way of non-
35 limiting examples.

As it will be apparent from the results described below, melusin plays a crucial role in the mechano-chemical signaling leading to a correct cardiomyocytes hypertrophy in response to pressure overload.

In the absence of melusin, cardiac hypertrophy is severely impaired and left ventricle undergoes dilation, thinning and displays reduced contractile capacity, thus becoming unable to withstand the biomechanical stress imposed by the high blood pressure.

Stimulating melusin function and signaling will expectedly improve cardiomyocytes hypertrophy preventing dilation and subsequent heart failure. Therapeutic strategies will, thus, involve expression or over-expression of melusin in failing hearts by gene transfer, use of drugs acting as agonists of melusin or of melusin downstream effector molecules.

Although several molecules such as the alfa Gq subunit, the phospholipase C beta, the protein kinase C, calcineurin, NF-AT3, Ras, Raf-1, ERK1/2, Jnk and p38MAP kinases, the phosphoinositide kinase 3 and the STATs (see discussion above) are potential targets for drugs aimed to stimulate heart hypertrophy, all these proteins have the great disadvantage of being ubiquitously expressed in most, if not all, tissues. A drug regulating any of these proteins will, thus, unavoidably cause deleterious side effects. Melusin, being a muscle specific protein, will not present this problem and, on the contrary, represents an ideal target molecule for such type of drugs. A second important advantage of melusin over other proteins involved in the heart hypertrophy is its role as mechanosensor, which places melusin in the very early steps of the biochemical signaling cascade triggering the hypertrophic response. Thus, regulating melusin

function allows a very specific control of the heart response.

The non-human transgenic animals in which the melusin gene is inactivated by homologous recombination or by genetic approaches different from homologous recombination represent a unique animal model for testing drugs aimed to prevent heart failure. In fact, these animals do not show functional heart defects in basal conditions during their lifespan. Heart failure become apparent only when transgenic animals and more preferably melusin-null transgenic mice are exposed to chronic hypertensive conditions. In a preferred embodiment the hypertensive conditions are determined by surgical constriction of the aorta, pharmacological treatment with hypertensive drugs or high sodium diet. In these conditions, the transgenic mice develop heart dilation and failure with a relatively slow kinetics (within 4 weeks), a time course much slower compared to that shown by other animal models for dilated cardiomyopathy (Arbet et al 1997; Hirota et al 1999; Badorff et al 2002) allowing to more accurately test drugs aimed to prevent cardiac failure.

The preparation of a non-human transgenic animal – preferably melusin-null transgenic animal – comprises essentially the steps of: i)preparing a genomic DNA construct abrogating melusin expression and suitable for homologous recombination event; ii) use of such DNA construct to induce homologous recombination in embryonic stem cells; iii) use of stem cells carrying an inactivated melusin gene to generate a chimeric embryo; iv) selecting animals heterozygote and homozygote for the melusin mutation by breeding the chimeric animals with different mouse strains.

A transgenic animal for melusin is an animal in which the expression of the melusin protein has been

altered/modified either in a positive or negative direction by stable or transient introduction in some or all cells of the animals of molecules capable to modify melusin expression at transcriptional,
5   translational or post-translational level.

As a non limiting example a DNA construct coding for an antisense melusin transcript can be used. Expression of such DNA construct is directed by a cardiac specific promoter such as the promoter of the
10 α-myosin heavy chain. This construct is introduced in fertilized oocytes that are then reimplanted in the uterus of foster mothers in order to generate non human transgenic animals that express either none or reduced level of melusin in heart. A second non limiting
15 example consists in the use of vectors coding short duplex RNAs of 21-23 nt capable to silence genes containing homologous sequences(Hasuwa et al., 2002).

The cardiac pathology displayed by the melusin-null transgenic mice described in the present invention
20 indicates that melusin is required to sustain the compensatory hypertrophic response when the heart is exposed to pressure overload. It is thus concluded that the inhibition of melusin function by natural or synthetic compounds can lead to cardiac failure and
25 dilation in animals carrying wild type melusin genes and exposed to hypertensive conditions.

EXAMPLES.

EXAMPLE 1. PRODUCTION OF MELUSIN-NULL TRANGENIC MICE AND MOLECULAR CHARACTERIZATION.

30 To investigate the role of melusin in integrin function the inventors have generated a mutation in mice (inbred 129SV strain) that abrogate melusin expression. Using the murine cDNA (Brancaccio et al. 1999; GenBank AF140691) the present inventors isolated
35 a genomic fragment of 14,8 Kb encompassing four exons

at the 5' end of the melusin gene. Partial characterization by restriction map and sequencing indicated that the first exon contains the ATG start codon. A PstI fragment containing exons 1 to 4 was replaced with a cassette containing IRES sequences linked to the LacZ gene followed by the neomycin resistance gene driven by a PGK promoter (Figure 2). This construct, which has two arms of 4.1 and 5 Kb homologous to the endogenous gene, was electroporated in embryonic stem R1 cells from male 129SV inbred mice. Different clones in which homologous recombination occurred have been identified by Southern blot analysis (Figure 3). Since the melusin gene is located on the X chromosome (Brancaccio et al 1999) and the ES R1 cells are of male origin, a single homologous recombination event was sufficient to inactivate the melusin gene (Figure 3). After injection of the mutant ES cells into blastocysts and implant in the uterus of a foster mother, chimeric mice in which the genetically modified cells have colonized the germ line were obtained. Chimeras were than breed with 129SV mice to obtain melusin-null 129SV mice.

The melusin-null mice are viable and fertile and do not show appreciable muscle or heart defects up to 18 months of age.

The successful inactivation of the melusin gene was demonstrated by analysis of protein expression both in heart and skeletal muscles of mutant mice (Figure 4). These data indicate, thus, that melusin is not required for muscle and heart development.

EXAMPLE 2. ROLE OF MELUSIN IN CARDIAC HYPERTROPHY.

Basal cardiac morphology and performance was investigated by echocardiography and cardiac catheterization and found to be comparable in wild type and melusin-null mice.

As shown in figure 5A echocardiography allowed to measure left ventricle end-diastolic (LVEDD), end-systolic diameter (LVESD), interventricular septum thickness in end diastole (IVSD), left ventricle
5 posterior wall thickness in end diastole (LVPWD) and fractional shortening (FS) (figure 5C). Left ventricle mass (LVW/BW) was also measured as hypertrophy parameter and found to be comparable in melusin-null and control mice (figure 5B). In addition the pressure
10 developed by the left ventricle was directly measured by catheterization with a micro manometer and reported as dP/dt (figure 5D). Thus the absence of melusin does not affect cardiac function under physiological conditions.

15 Melusin deficiency, however, affects cardiac muscle function when hearts are exposed to pressure overload. To analyze the ability to respond to biomechanical overload, melusin-null hearts were subjected to chronic hypertension realized by surgical
20 constriction of the transverse aorta as described below and in Figure 6.

Mice were anesthetized by injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). After midline sthernotomy, the aortic arch is constricted
25 between truncus anonimus and left carotid artery with 8-0 silk tied against the vessel and a blunted 27-gauge needle, which is promptly pulled out thereafter. In the control group the same surgical procedures are performed without constricting the aortic arch. Both
30 wild type and melusin-null mice were subjected to the above surgical procedure. 7 days after surgery the degree of hemodynamic overload was evaluated as systolic pressure gradient measured by selective cannulation of left and right carotid arteries (Lembo
35 et al. 1996)

After these hemodynamic evaluations, the mice are weighed, hearts excised and evaluation of cardiac hypertrophy obtained with left ventricular weight/body weight ratio.

5   While wild type mice develop an overt compensatory cardiac hypertrophy 7 days after surgery, melusin-null mice show only a very modest hypertrophy, evaluated by left ventricular/body weight ratio (Figure 7).

To better characterize the evolution of the left
10 ventricle remodeling during chronic pressure overload, melusin-null mice were subjected to TAC (transverse aortic constriction) for a period of 4 weeks and examined by serial echocardiographic analysis during this period. Cardiac structure and function were
15 evaluated not invasively with transthoracic echocardiography in basal condition and after 2 and 4 weeks from TAC. All measurements were determined in a short axis view at the level of papillary muscles.

As expected, wild-type mice showed increased
20 interventricular septum thickness and reduced end-distolic left ventricular diameters. In contrast, after 7 days of TAC, melusin-null mice developed only modest thickening of ventricular walls and a significant chamber enlargement (Figure 8). After 2 weeks from TAC,
25 melusin-null mice showed a further enlargement of left ventricular chamber as compared to that observed in wild-type mice (Figure 11). After 4 weeks, left ventricular dilation was even more evident and associated with a marked deterioration of contractile
30 function, as detected by the severe impairment of fractional shortening (Figure 11). Finally, the lethality rates at 4 weeks from TAC were greater in mutant as compared to wild-type mice (53,3% vs 30,7%).

The absence of melusin results, thus, in reduced
35 cardiac hypertrophy and promotes the left ventricle

dilation when hearts are exposed to increased blood pressure. This condition is, thus, accelerating the onset of the defective cardiac response.

EXAMPLE 3. MECHANOSENSOR ROLE OF MELUSIN IN THE HEART.

5 To test whether melusin is involved in heart hypertrophy in response to stimuli different from pressure overload, the inventors also tested the cardiac response in melusin-null mice after chronic administration of phenylephrine or angiotensin II at

10 sub-pressor doses which do not increase blood pressure.

Chronic administration of sub-pressor doses of phenylephrine (100 mg/kg/day) or angiotensin II (0,1 mg/kg/day) (Harada et al, 1998) was obtained by subcutaneous implantation of osmotic mini-pump (Alza

15 Corp.) delivering the above doses of phenylephrine or angiotensin II for 21 days. In these experimental series control groups were treated with vehicle alone.

To verify that chronic agonists infusion does not alter blood pressure homeostasis, blood pressure

20 profile was evaluated by radio-telemetric measurement realized through implantation of a commercially available device into the femoral artery and acquisition of the telemetered pressure signal in a dedicated, computed analysis system (Data Sciences

25 International).

Cardiac hypertrophy was evaluated by left ventricular/body weight ratio. The results of these experiments indicate that melusin-null mice exposed to sub-pressor doses of phenylephrine or angiotensin II

30 develop left ventricle hypertrophy in a manner not significantly different from wild type mice (Figure 9).

These results altogether indicate that melusin is involved in the hypertrophic response to pressure overload (see Example 2), but is not required in the

35 response to trophic factors such as phenylephrine or

angiotensin II. This, thus, strongly points for a mechanosensor role of melusin in heart.

EXAMPLE 4. THE LACK OF MELUSIN IMPAIRS GSK3β PHOSPHORYLATION.

5   To investigate the impact of melusin on cardiac intracellular signaling triggered by biomechanical stress, phosphorylation of signaling proteins reported to be involved in cardiac hypertrophy (Aoki and Izumo 2001; Hunter and Chien 1999; Hardt and Sadoshima, Circ
10   Res. 2002) was analyzed.

Representative experiments are shown in Figure 12. Wild-type (WT, empty bars) and melusin-null mice (KO, filled bars) were subjected to TAC for 10 min or to sham (S) operations as controls. Left ventricle protein
15   extracts were analyzed by western blotting with antibodies to phosphorylated signaling molecules.

In particular the inventors found that glycogen synthase kinase 3beta (GSK3β) was differentially phosphorylated in wild type versus melusin-null mice.
20   As shown in Figure 12c, GSK3β was strongly phosphorylated at serine 9 residue 10 minutes after TAC in wild-type mice consistently with the hypothesis that this signal was triggered in response to a mechanical event. However, in melusin-null mice the degree of
25   GSK3β serine 9 phosphorylation was severely reduced (Figure 12c).

Since AKT is a major kinase regulating GSK3β serine 9 phosphorylation the inventors analyzed the phosphorylation state of this kinase. While AKT is
30   rapidly phosphorylated in response to TAC in wild type mice, this response was strongly reduced in melusin-null mice (Figure 12d).

GSK3β is involved in multiple signaling pathways and is a well known target of insulin receptor
35   signaling (Cohen and Frame 2001). The inventors then

tested whether the lack of melusin could affect GSK3β phosphorylation in response to insulin. Western blot analysis of heart extracts from mice treated for 10 and 20 min with insulin – administered with IP injections of 2IU – showed that GSK3β was phosphorylated at comparable level in both mice genotypes (Figure 12e).

Since attenuation of cardiac hypertrophy in melusin-null mice was observed after 7 day TAC, the inventors then tested GSK3β signaling at this point in time. Interestingly GSK3β serine 9 phosphorylation was reduced in melusin-null mice versus wild type after 7 days of TAC (Figure 12f). In addition kinase activity was increased as predicted by the inhibitory action of serine 9 phosphorylation (Figure 12g). Thus altered GSK3β signaling is persistent in melusin-null mice exposed to 7 day TAC.

These data indicate that the lack of melusin selectively impairs left ventricular AKT and GSK3β phosphorylation in response to biomechanical stress.

EXAMPLE 5.   ISOLATION   OF   MELUSIN-AGONIST   ORGANIC COMPOUND.

In order to identify melusin agonists, molecules capable of activating melusin function have to be identified. Based on the previously published (Brancaccio et al 1999) and present data, integrins bind melusin and trigger its activation. Thus, peptides or organic compounds binding to melusin and interfering with melusin-integrin binding are expectedly good candidates as melusin agonists by mimicking the integrin-induced melusin activation. Such melusin agonists can be identified from a large library of peptide and peptide-like compounds by using the techniques of high-throughput screening using a well-defined assay for the detection of such agonists

To this end, an ELISA assay can be used in which purified recombinant melusin, or fragments of the protein is adsorbed on the surface of microtiter wells are incubated with cell extracts containing integrin to 5 allow integrin-melusin binding. To this mixture specific compounds are added to select molecules capable to bind melusin and prevent integrin binding. A similar procedure has already been used to select compound capable to interfere with integrin function in 10 other cellular systems (Ambroise et al. 2002)

The following ELISA protocol can be used. Fusion proteins consisting GST (Glutathione S-trasferase) fused to the full-length human melusin and/or a fragment from amino acid residues 149-350 in the C- 15 terminal region, containing the integrin binding site (Brancaccio et al 1999) are purified by affinity chromatography on glutathione-Sepharose 4B. Purified fusion proteins are adsorbed on microtiter wells according to standard procedures and used as ligands 20 for integrin binding. COS cell extracts are used as source of beta1 integrin heterodimers.

Briefly COS cells are washed twice with cold PBS and extracted in TBS (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM NaVO4, 10 mM NaF, 10 µg/ml leupeptin, 4 25 µg/ml pepstatin and 0.1 TIU/ml aprotinin) 0.5% Nonidet P-40 plus 1 mM Ca^{2+}, or with TBS, 0.5% Nonidet P-40 plus 5 mM EDTA to solubilized membrane proteins. 100 µl of cell extracts containing 2 mg of proteins/ml are incubated overnight at 4°C in wells coated with GST- 30 melusin, GST-melusin(aa149-350) and GST alone (as control). After washing, integrin binding is detected with the TS2/16 monoclonal antibody followed by peroxidase-conjugated anti-mouse antibody. The compounds capable to interfere with integrin-melusin 35 binding will be added at increasing concentrations

together with the COS cell extract during the incubation with the GST-melusin fusion protein.

As source of compounds capable to interfere with melusin-integrin binding, random peptides phage display
5 library can be used (Ladner and Ley 2001). In such libraries random oligonucleotide sequences coding for short amino acid sequences (8-18 residues) are inserted in the coding sequence of the phage coat proteins. The resulting phage displays on its surface the random
10 peptide sequence to be selected for its binding capacity. The phage population displaying the random peptides is allowed to interact with recombinant melusin adsorbed on the surface of microtiter wells before incubation with integrins. Phages interfering
15 with integrin binding are isolated and the peptide sequence coded for by the inserted random oligonucleotide will be determined by DNA sequencing.

As an alternative source of organic compounds capable to bind melusin interfering with integrin
20 binding combinatorial chemistry libraries are used (Floyd et al. 1999; Ambroise et al. 2002; Toogood 2002).

The peptide sequences isolated with the procedure described above will be tested for their ability to
25 trigger in vitro cardiomyocyte hypertrophy. For this test melusin-null cardiomyocytes derived from the trangenic animals of the invention are used to define the specificity of the isolated compound toward melusin. Such compound are ineffective on melusin-null
30 cardiomycytes while they should be active on wild type cells.

To allow penetration in the cells, the peptides are coupled to trojan peptides (Derossi et al 1998) that allow spontaneous and efficient intracellular
35 delivery.

Active peptides are also used to develop structural analog organic compounds more suitable for in vivo treatment.

Genetically modified mice models developing, either spontaneous or pressure overload-induced, dilated cardiomyopathy (for review see Chien, 1999) are treated by delivering peptide analogs with subcutaneous implantation of infusion mini-pumps and analyzed for their cardiac function and morphology to monitor the in vivo potential therapeutic activity of the compounds.

The same strategy is applied with molecules acting downstream of melusin in controlling the cardiac hypertrophy response. Using different experimental approaches, including co-immunoprecipitation, affinity chromatography and the two hybrid test, proteins binding to melusin and functioning as downstream transducers of the mechanochemical signal leading to hypertrophy can be identified (between step 2 and 3 in figure 10). Once these molecules are identified and characterized, the same strategy described above can be applied to select agonists that boost the activation of such proteins acting downstream of melusin. Such drugs are tested in melusin-null transgenic mice for their ability to rescue left ventricle dilation observed in these animals after TAC.

EXAMPLE 6. MELUSIN GENE-THERAPY TO PREVENT AND TREAT HEART DILATION AND FAILURE

As discussed above an alternative therapeutic strategy to prevent and/or cure heart dilation and failure can be achieved by inducing over-expression of melusin.

Adenovirus constructs and/or other viral vectors such as lentiviral vectors have been proved efficient vector for gene delivery in experimental heart pathologies (Wright et al 2001). Adenoviral vector

expressing the melusin gene are prepared according to the following protocol given as an example. Lentiviral vectors can be prepared by similar procedures as well.

Human melusin cDNA (GenBank AF140690) is cloned in a shuttle vector (pAdTrack-CMV) containing GFP marker. 100-500 ng of the resultant plasmid is linearized by digestion with PmeI restriction endonuclease, and after digestion extracted with phenol-cloroform treatment, precipitated with etanol and resuspended in 6 µl of deionised water. PmeI-digested shuttle plasmid is co-transformed with 100ng of adenoviral backbone vector (pAdEasy-1) by electroporation in BJ5183 E.Coli cells. Transformed E.Coli cells are resuspended in 500 µl of L-broth, plated on 3 LB kanamycin plates and grown overnight at 37°C. 10-20 colonies are picked up and grown in 2 ml L-broth containing 25 µg/ml kanamycin for 10-15 hours. DNA minipreps are performed with conventional methods and supercoiled plasmids are digested with PacI restriction endonuclease. Candidate clones yield after digestion a large fragment of about 30 kb and a smaller one of 3 or 4,5 kb. Recombinant adenovirus is retransformed in E. Coli and is purified using commercially available purifications kits. 4 µg of recombinant adenoviral DNA is then linearized wth PacI restriction endonuclease, precipitated with ethanol, resuspended in 20 µl of sterile water and used for transfection of $2x10^6$ 293 cells (E1-transformed human embryonic kidney cells) at 50-70% confluence. Transfection and viral production is monitored by the fluorescent protein GFP expression. Cells are scraped with a rubber policeman at 7-10 days post-transfection and collected in 50 ml tubes, then spinned in a centrifuge and resuspended in 2 ml sterile PBS. Cells are frozen in dry ice-methanol bath and are thawed in a 37°C water bath and then vortexed. Cells are frozen and

thawed for a total of 4 cycles. Then samples are spinned briefly and stored at -20°C. This viral supernatant is used to infect 50-70% confluent 293 cells in order to produce large amount of viral stocks.

5   The virus are collected when a third to half of the cells are detached. It is possible to confirm the virus presence using PCR and western blot analysis. Viral titer is measured by counting green fluorescent 293 cells 18 hours after infection with various dilutions

10   of virus supernatant.

The adenoviral vector is delivered to animals following a catheter-based protocol for intracardiac injection according to Hajjar et al. (Hajjar et al 1998).

15   As proof of efficacy in vivo, the melusin adenoviral vector are tested for its ability to rescue cardiac dilation in melusin-null mice subjected to transverse aortic constriction. This procedure is also applied on different transgenic mouse models with

20   impaired hypertrophy response, or with spontaneous dilated cardiomyopathy.

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CLAIMS

1. Non-human transgenic animal, being transgenic for having altered melusin expression.

2. Non-human transgenic animal according to claim 1, characterized in that said altered melusin expression is performed by stable or transient modification of melusin expression at transcriptional, translational or post-translational level.

3. Non-human transgenic animal according to claim 1 or 2, characterized in that said altered melusin expression is an inactivation of melusin gene.

4. Non-human transgenic animal according to claim 3, characterized in that said gene inactivation is performed by genetic approaches.

5. Non-human transgenic animal according to claim 4, characterized in that said genetic approaches are selected from the group consisting of homologous recombination, antisense RNA or DNA and RNA or DNA interference approach.

6. Non-human transgenic animal according to any of the preceding claims, characterized in that said animal is a melusin-null transgenic animal.

7. Non-human transgenic animal according to any of the claims 1 to 6, characterized in that said animal is subjected to hypertensive condition.

8. Non-human transgenic animal according to claim 7, characterized in that said hypertensive condition is determined by surgical operation.

9. Non-human transgenic animal according to claim 8, characterized in that said surgical operation consists in surgical constriction of the transverse aorta.

10. Non-human transgenic animal according to claim 7, characterized in that said hypertensive condition

is determined by pharmacological treatment, preferably with hypertensive drugs.

11. Non-human transgenic animal according to claim 7, characterized in that said hypertensive condition is determined by high sodium diet.

12. Non-human transgenic animal according any of the preceding claims, wherein said animal develop at least impaired heart hypertrophy.

13. Non-human transgenic animal according any of the preceding claims, wherein said animal develop at least heart dilation.

14. Non-human transgenic animal according any of the preceding claims, wherein said animal develop at least heart failure.

15. Non-human transgenic animal according any of the preceding claims, wherein said animal is a mammalian.

16. Non-human transgenic animal according to claim 15, wherein the mammalian belongs to the murine genus(mus musculus).

17. Non-human transgenic animal according to claim 16, wherein said mouse belongs to the 129SV, C57Bl or 129SVxC57Bl strain.

18. Use of non-human transgenic animal according to any of the preceding claims for the selection of compounds pharmacologically active in the prevention and/or treatment of heart failure.

19. Use of non-human transgenic animal according to any of claims 1 to 17 for the study of heart pathologies, wherein said heart pathologies are selected from the group consisting of: heart failure, congestive heart failure, dilated cardiomyopathy, hypertensive cardiomyopathy, hypertrophic cardiomyopathy, heart infarct.

20. Cells derivable from the non-human transgenic animal according to any of claims 1 to 17 and having altered melusin expression.

21. Cells according to claim 20, characterized in that said cells carry a mutation inactivating melusin gene.

22. Cells according to claim 20 or 21, characterized in that said cells are lacking melusin expression.

23. Use of cells according to any claim 20 to 22 for the screening of compounds pharmacologically active for the prevention and/or treatment of heart failure.

24. Method for the preparation of a non-human transgenic animal according to claim 1 comprising essentially the steps of:

    i)   preparing a non-human transgenic parent animal carrying an inactivated melusin allele;

    ii)   breeding the parent transgenic animal with a non transgenic animal;

    iii)   selecting transgenic animals heterogyzote for the melusin gene mutation.

25. Method according to claim 24, further comprising the step of iv) breeding the heterozygote transgenic animals to select homozygote transgenic animals for the melusin gene mutation.

26. Non-human animals in which melusin function has been inhibited by the use of natural or synthetic compounds.

27. Use of the animal according to claim 26 to study the impaired cardiac hypertrophy.

28. Use of the animal according to claim 26 to study cardiac dilation.

29. Use of the animal according to claim 26 to study the heart failure.

30. Method for screening compounds able to interact with melusin binding proteins, said compounds being pharmacologically active in the prevention and/or treatment of heart failure, wherein said method comprises using melusin, fragments and/or derivatives thereof.

31. Method for screening compounds able to interact with melusin, said compounds being melusin agonists and pharmacologically active in the prevention and/or treatment of heart failure, wherein said method comprises using melusin, fragments and/or derivatives thereof.

32. Use of melusin, fragments and/or derivatives thereof for the manufacture of a medicament for the prevention and/or treatment of heart failure.

33. Use of melusin, fragments and/or derivatives thereof for the screening of compounds pharmacologically active for the prevention and/or treatment of heart failure.

34. Use according to claim 33, characterized in that said pharmacologically active compound is a melusin agonist.

35. Use according to claim 33, characterized in that said pharmacologically active compound is able to interact with melusin-binding proteins.

36. Use of a DNA vector for the manufacture of a medicament for use in the prevention and/or treatment of heart failure, said vector comprising a transgene coding for the melusin protein or fragments thereof and expressing said transgene in the myocardium.

37. Use according to claim 36, characterized in that said transgene comprises melusin cDNA or fragments thereof.

38. Use according to any claim 36 to 37, characterized in that said vector is an adenoviral vector or a lentiviral vector.

39. Pharmaceutical compositions comprising melusin, fragments and/or derivatives thereof for the prevention and/or treatment of heart failure.

1/11

Figure 1

```
     1         10        20        30        40        50        60
Mo MSLLCYNKGCGQHFDPNTNLPDSCRYHPGVPIFHDALKGWSCCRKRTVDFSEFLNIKGCTVGLHCAEKL
   |||||  |||||||||||||||||||| :||||||||||||||||||||||||||||||||||||||: | ||||||
hu MSLLCRNKGCGQHFDPNTNLPDSCCHHPGVPIFHDALKGWSCCRKRTVDFSEFLNIKGCTMGPHCAEKL

     70        80        90        100       110       120       130
mo PEVPPQPEGPATSS-LQEQKPLNTIPKSAETLFRERPKSEMPPKLLPLLISQALGVALEQKELDQEPGA
   ||:| |||||||||| |||||||||:|||||||||| |||||||:| ||||| |||||  :|||||||||||
hu PEAP-QPEGPATSSSLQEQKPLNVIPKSAETLRRERPKSELPLKLLPLNISQALEMALEQKELDQEPGA

     140       150       160       170       180       190       200
mo GLDNSLIWTGSSCQNPGCDAVYQGPESDATPCTYHPGAPRFHEGMKSWSCCGIQTLDFGAFLAQPGCRV
   |||  |||  |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
hu GLD-SLIRTGSSCQNPGCDAVYQGPESDATPCTYHPGAPRFHEGMKSWSCCGIQTLDFGAFLAQPGCRV

     210       220       230       240       250       260       270
mo GRHDWAKQLPASCRHDWHQTDSVVVLTVYGQIPLPAFNWVKASQTELHVHIVFDGNRVFQAQMKLWGVI
   |||||:|||||||||||||||:||:|||||||||||||||||||||||||||||||||||||||||||||
hu GRHDWGKQLPASCRHDWHQTDSLVVVTVYGQIPLPAFNWVKASQTELHVHIVFDGNRVFQAQMKLWGVI

     280       290       300       310       320       330       340
mo NVEQSSVSLMPSRVEISLVKADPGSWAQLEHPDSLAEKARAGVLLEMDEEESEDSDDDLSWTEEEDEEE
   ||||||| |||||||||||||||||||||||||||| || ||||||:|||||||:|||||||||||||||  |||
hu NVEQSSVFLMPSRVEISLVKADPGSWAQLEHPDALAKKARAGVVLEMDEEESDDSDDDLSWTEE--EEE

     350
mo EEAMGE
   ||||||
hu EEAMGE
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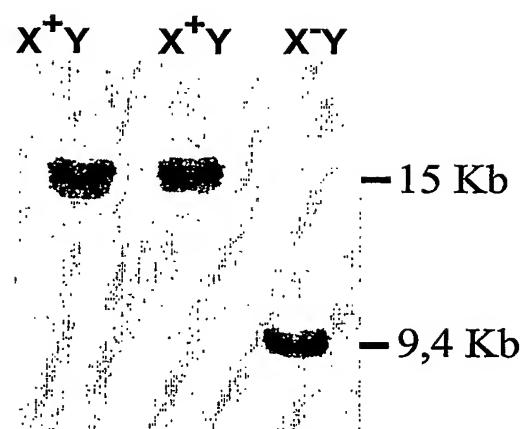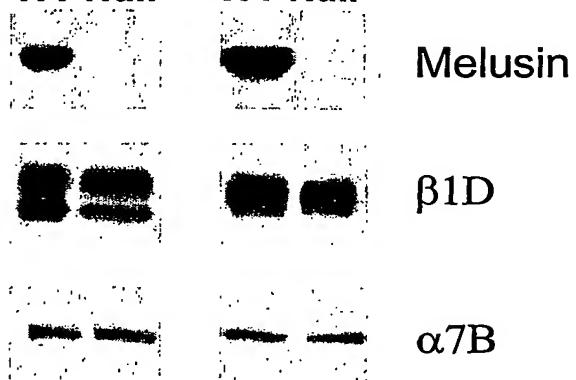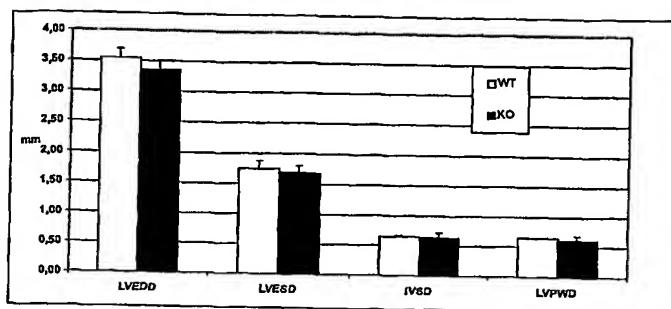
Figure 2

Figure 3

b

Figure 4

c

Figure 6

Figure 7

Figure 8
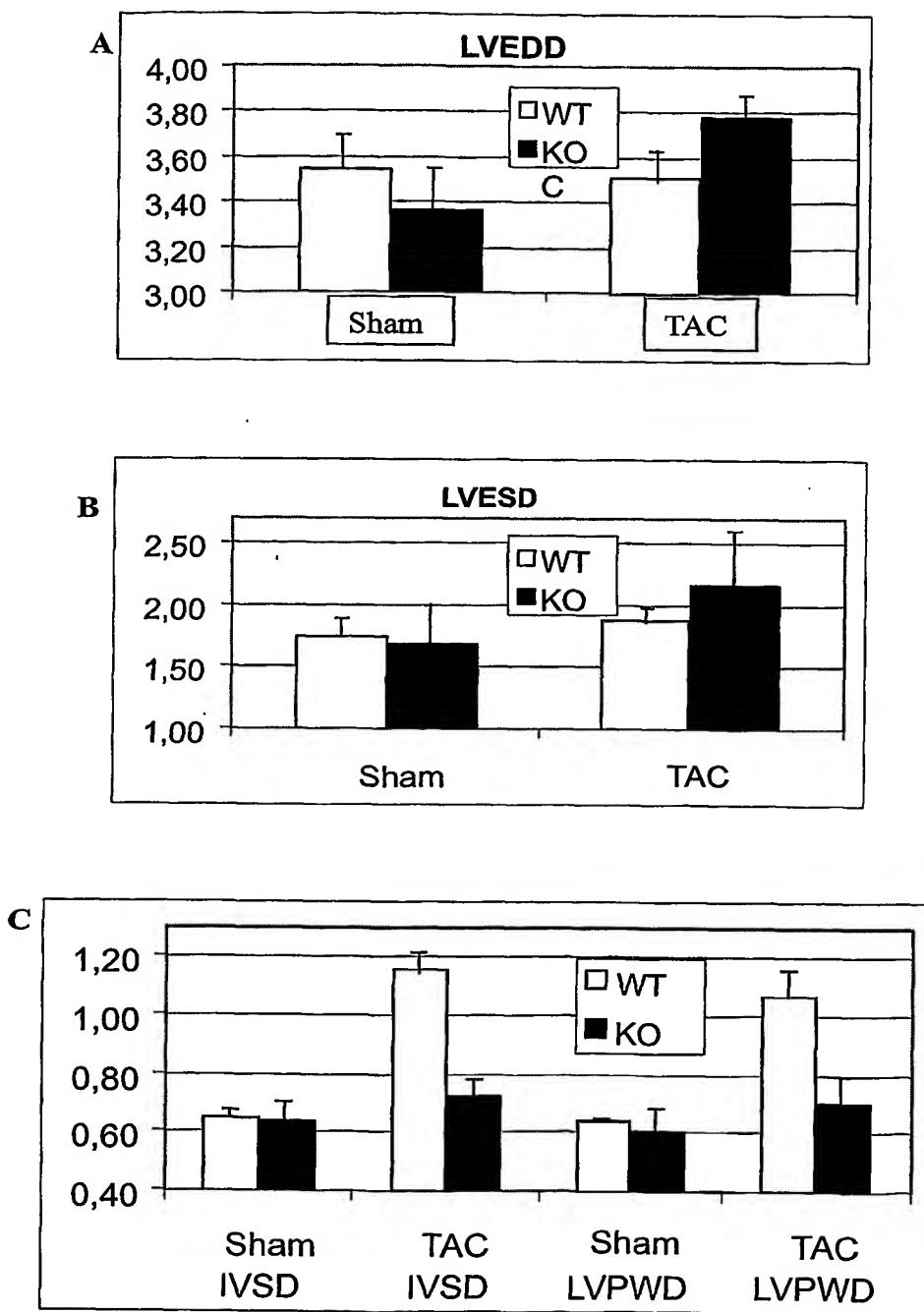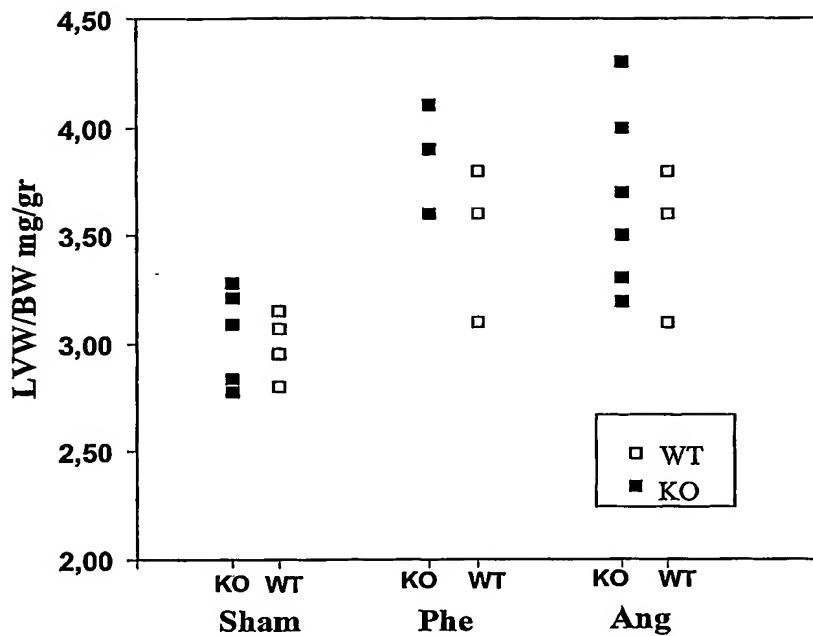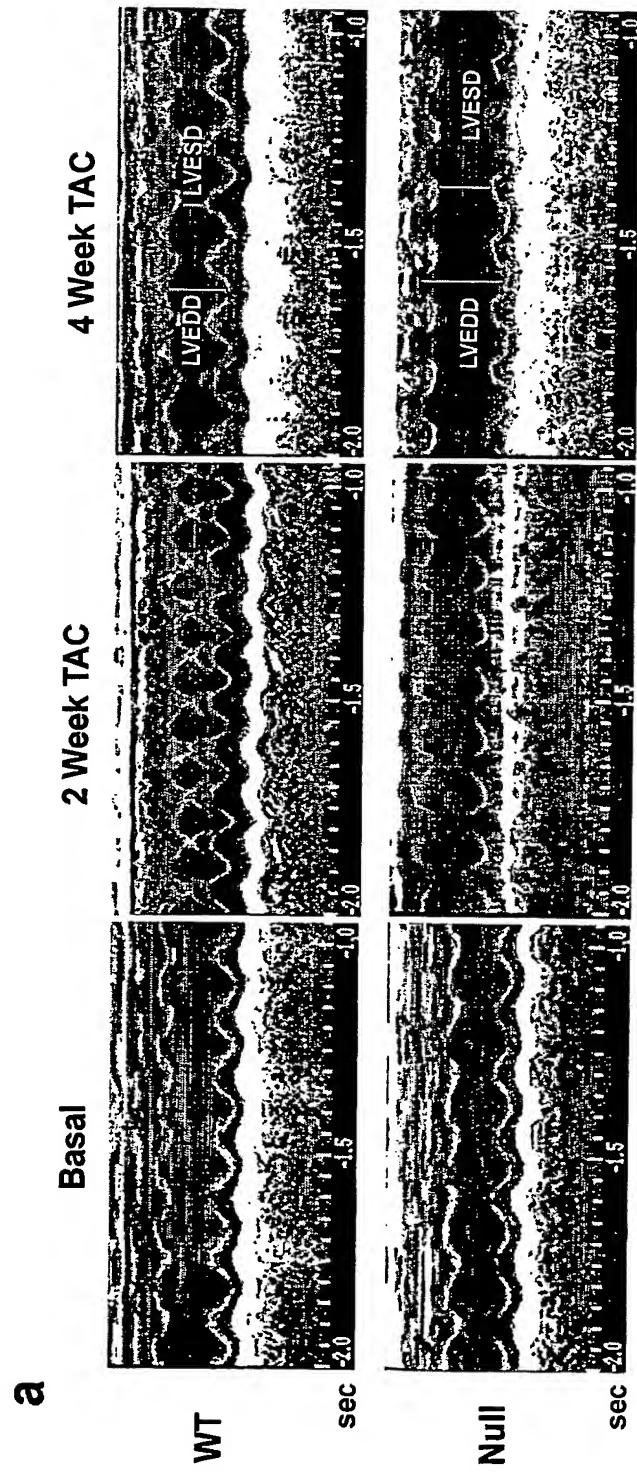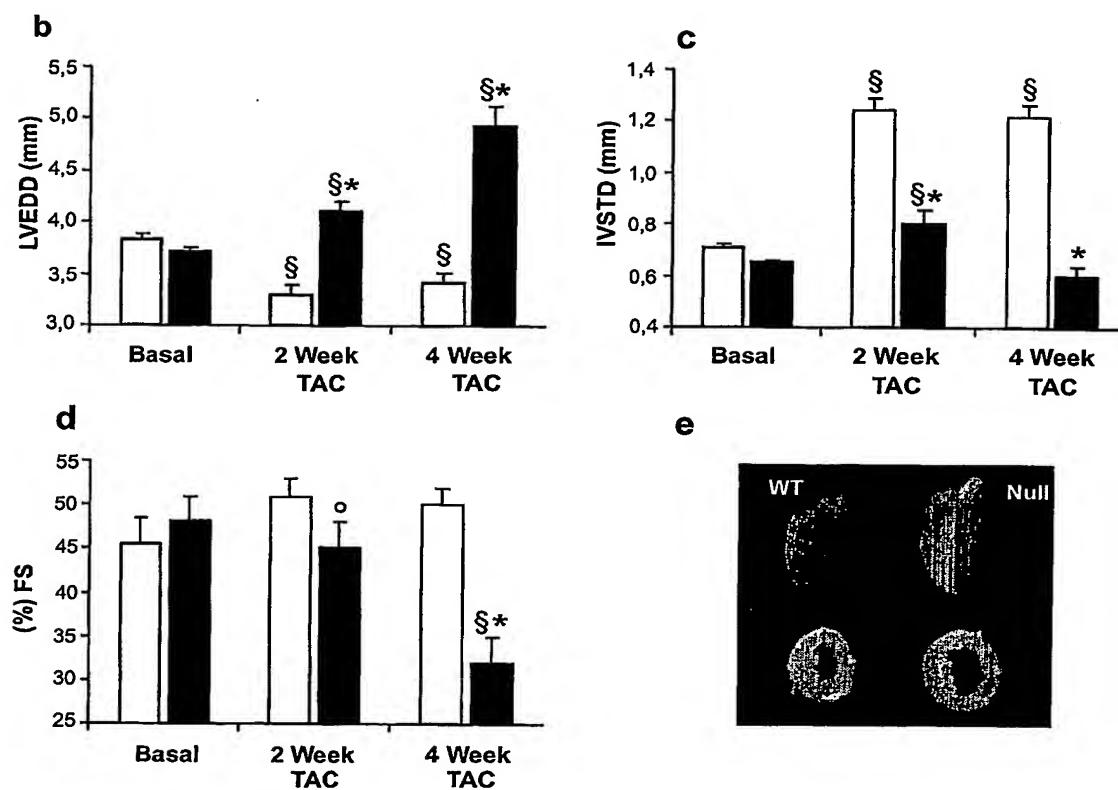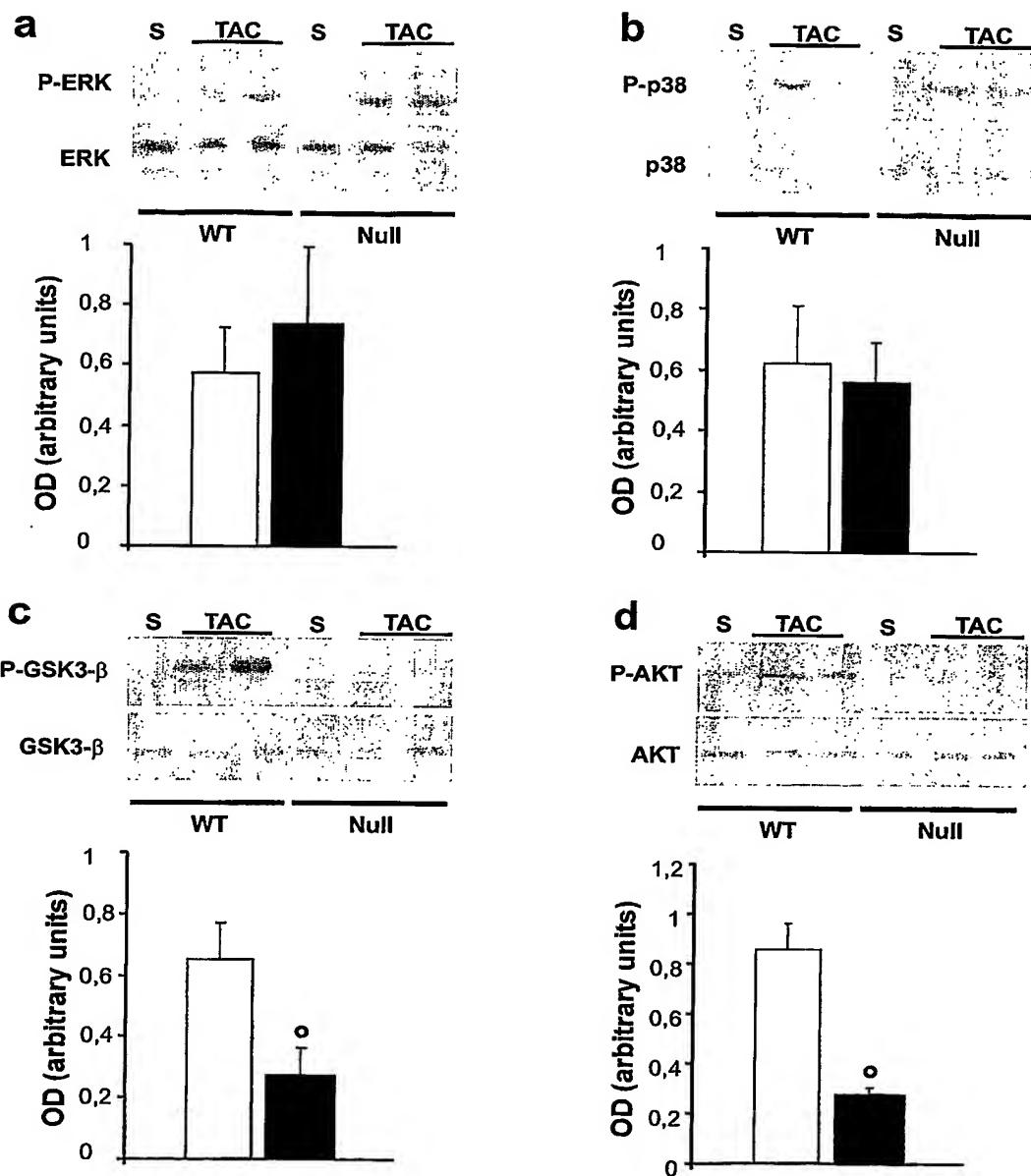
A LVEDD

B LVESD

C

Figure 9

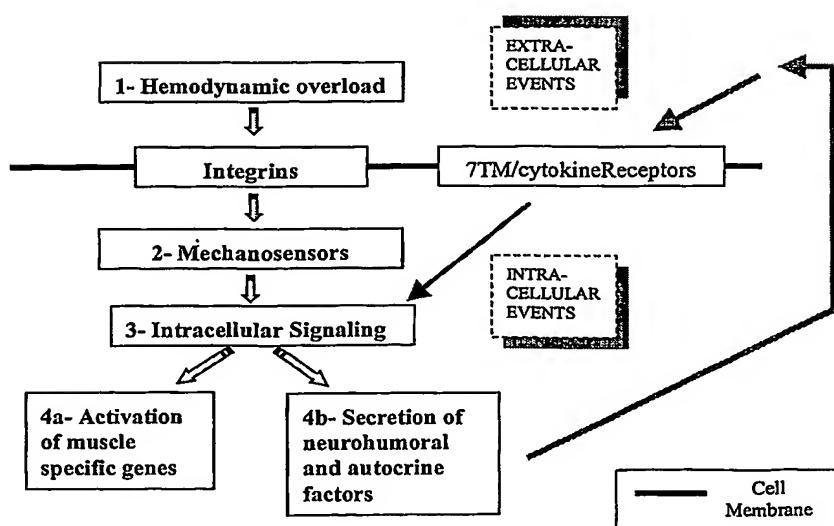Figure 10

Figure 11

Figure 11

b

c

d

e

Figure 12

Figure 12
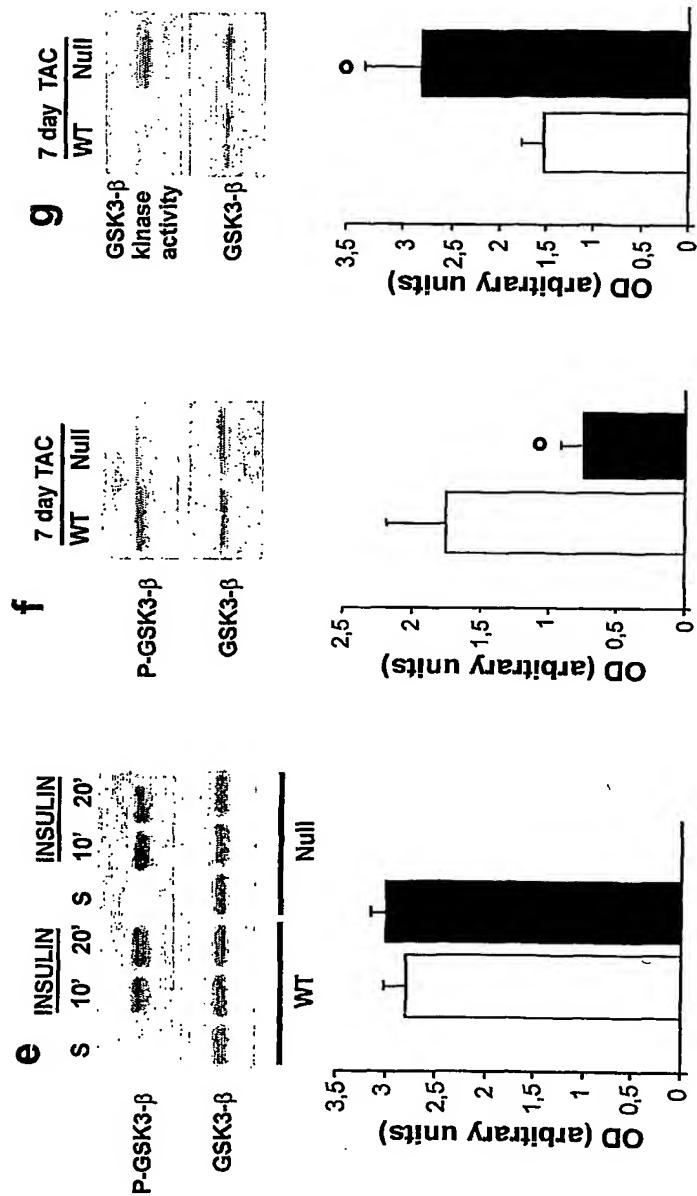
A. CLASSIFICATION OF SUBJECT MATTER

IPC 7   A01K67/027   G01N33/50   A61K48/00   A61K38/17   //C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   C07K   A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BRANCACCIO M. ET AL.: "Melusin is a new muscle-specific interactor for beta1 integrin cytoplasmic domain." JOURNAL OF BIOLOGICA CHEMISTRY, vol. 274, no. 41, - 8 October 1999 (1999-10-08) pages 29282-2928, XP002240486 cited in the application the whole document --- -/--	1-28, 30-39

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 May 2003	30/05/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Galli, I

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
T	BRANCACCIO M. ET AL.: "Melusin, a muscle-specific integrin beta1-interacting protein, is required to prevent cardiac failure in response to chronic pressure overload." NATURE MEDICINE, vol. 9, no. 1, January 2003 (2003-01), pages 68-75, XP002240487 the whole document	1-28, 30-39

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:   26—29
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.:   26-29

Claims 26-29 relate to non-human animals in which melusin function has been inhibited by compounds. In other words, what is claimed here are animals treated with compounds. Neither the claims nor the description disclose what these compounds could be, so a search for novelty (let alone inventive step) is not possible.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.